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Phycobilisome rod mutants in *Synechocystis* sp. strain PCC6803

Bettina Ughy† and Ghada Ajlani

Département de Biologie Joliot-Curie, SBFM, CEA Saclay, F-91191, Gif-sur-Yvette, France

Correspondence
Ghada Ajlani
gajlani@cea.fr

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The phycobilisome is a large pigment-protein assembly that harvests light energy for photosynthesis. This supramolecular complex is composed of two main structures: a core substructure and peripheral rods. Linker polypeptides assemble phycobiliproteins within these structures and optimize light absorption and energy transfer. Mutations have been constructed in three rod-linker-coding genes located in the *cpc* operon of *Synechocystis* sp. strain PCC6803. The *cpcC1* gene encoding the 33 kDa linker is found to be epistatic to *cpcC2* encoding the 30 kDa linker, indicating a specific role for each of these two linkers in rod growth. This corroborates studies on the sequential degradation of phycobilisomes upon nitrogen starvation. Three allelic mutants affecting *cpcC2* revealed a polar effect of commonly used cassettes (*aphI*, *aadA*) on the operon steady-state transcripts and an effect of rod linker availability on the amount of phycocyanin incorporated in the phycobilisome. This led to the proposal that regulation of rod length could occur through processing of transcripts upstream of the *cpcC2* gene.

INTRODUCTION

Phycobilisomes are sizeable (5–10 MDa) biliprotein complexes that harvest light for photosynthesis in cyanobacteria and red algae (Sidler, 1994). Although a phycobilisome (PBS) is composed of hundreds of biliproteins and fewer linker polypeptides (de Marsac & Cohen-Bazire, 1977), light energy absorbed anywhere within the particle is efficiently transferred towards a specific biliprotein which functions as a terminal-energy emitter (Glazer, 1989). A phycobilisome consists of six rods radiating from a core associated with the thylakoid membrane. Phycocyanin (PC) is a biliprotein (phycobiliprotein) found in the rods, whereas the core is composed of allophycocyanin (AP). Some cyanobacteria and red algae contain different biliproteins (i.e. phycoerythrin) in their rods. PC and AP carry the same blue chromophore called phycocyanobilin, the properties of each phycocyanobilin being strongly influenced by its protein environment. Rods containing only PC are composed of two or three (depending on the strain) disc-shaped hexamers of PC, in which a monomer is an (αPC, *β*PC) heterodimer. Specific rod linkers (L_R) assemble the PC hexamers into rods and tune their electronic properties in order to optimize directional energy transfer (Glazer, 1989).

As the PBSs in cyanobacteria constitute a substantial fraction of the total soluble proteins (50 %), their degradation can supply amino acids for the synthesis of essential proteins under nutrient-limited conditions (Allen & Smith, 1969). Indeed, the PBS is degraded in a specific and orderly manner during nitrogen starvation of non-diazotrophic cyanobacteria (Yamanaka & Glazer, 1980). This process, known as bleaching or PBS trimming, requires the *nblA* gene, which is induced and necessary for PBS trimming under nitrogen starvation (Collier & Grossman, 1994; Luque *et al.*, 2003). During PBS trimming, PC hexamers and associated linkers most distal to the core are degraded first (Richaud *et al.*, 2001; Yamanaka & Glazer, 1980). Another type of PBS trimming occurs under high-light conditions and does not involve *nblA* (Collier & Grossman, 1994; Garnier *et al.*, 1994; Lönneborg *et al.*, 1985).

PBSs are not required for phototrophic growth (Ajlani & Vernotte, 1998; Bruce *et al.*, 1989) so mutations affecting different linkers are of special interest because they provide the opportunity for observing PBS biogenesis in normal growth conditions. Understanding PBS assembly has been enhanced by mutational analysis of *Synechococcus* sp. strain PCC7002, but this strain contains only two PC hexamers and two linkers per rod. The phycobilisome of *Synechocystis* sp. strain PCC6803 consists of a three-cylindrical core from which six rods radiate, each rod being composed of three stacked PC hexamers and three rod linkers (Elmorjani *et al.*, 1986). Most of the rod-subunit-encoding genes are clustered in the *cpc* operon. In *Synechocystis* sp. strain PCC6803, this operon contains five genes: *cpcB* and *cpcA* encode the *β*PC and αPC subunits, respectively, while *cpcC2*,

†Present address: Institute of Plant Biology, BRC, Temesvári Krt. 62, H-6701, Szeged, Hungary.

Abbreviations: AP, allophycocyanin; FNR, ferredoxin $NADP^+$ reductase; L_x, linker polypeptide located at position x of the phycobilisome, where x can be R (rod), RC (rod core) or CM (core membrane), linkers with identical location are distinguished by a superscript just above the x indicating their molecular mass; PC, phycocyanin; PBS, phycobilisome.

cpcC1 and *cpcD* encode the rod linkers L_R^{30}, L_R^{33} and L_R^{10}, respectively. Two independent genes (*cpcG1* and *cpcG2*) encode the rod-core linker (L_{RC}) that attaches the proximal PC hexamer to the core (Cyanobase: http://www.kazusa.or.jp/cyano/Synechocystis/).

Using interposon mutagenesis, we have constructed and characterized deletion mutants in the three rod-linker genes located in the *cpc* operon of *Synechocystis* sp. strain PCC6803. Characterization of the PBS assembly process in the mutants yielded a scheme for PBS-rod biogenesis in *Synechocystis* sp. strain PCC6803 in which the insertion of L_R^{30} is excluded in the absence of L_R^{33}. This result differs from studies with mutants of *Synechococcus* sp. strain PCC7942 lacking rod-linker genes, in which it has been proposed that L_R^{30} and L_R^{33} could occupy interchangeable positions within the rods (Bhalerao *et al.*, 1991, 1993).

We demonstrated, by monitoring steady-state RNA levels in the mutants, that the cassette insertion affected gene expression in its vicinity, regardless of orientation. This polar effect provided us with strains containing different levels of the rod linkers, and whose absorption spectra showed that the PC level incorporated in the PBS varied proportionally. This result leads to the proposal that transcriptional regulation of the rod-linker genes adjusts the light-harvesting capacity of the photosynthetic apparatus by modulating the PBS rod length.

METHODS

Bacterial growth conditions. Wild-type and mutant strains of the cyanobacterium *Synechocystis* sp. strain PCC 6803 were grown photoautotrophically in an illuminated orbital incubator at 32 °C in a CO_2-enriched atmosphere and under continuous light (40 μE m^{-2} s^{-1}). Allen's medium (Allen, 1968) was modified as follows: 30 μM ferric citrate, 3 μM disodium EDTA, 30 mM sodium nitrate, 250 μM potassium phosphate, 250 μM magnesium sulphate, 250 μM calcium chloride, 200 μM sodium carbonate, 10 mM sodium bicarbonate and microelements as in Allen's medium. All chemicals were purchased from Sigma. For growth on plates, the above medium was supplemented with 1·5 % (w/v) Difco Bacto agar and 2 mM sodium thiosulfate. Plates were incubated at 30 °C under continuous light (30 μE m^{-2} s^{-1}). Where appropriate, media were supplemented with 25–50 μg kanamycin ml^{-1}, 25–50 μg spectinomycin ml^{-1} and 5–10 μg streptomycin ml^{-1}. *Escherichia coli* strain DH5α was used as plasmid host for cloning. Strains and plasmids used in this work are listed in Table 1.

Nucleic acid purification and reactions. Genomic DNA was isolated from *Synechocystis* sp. strain PCC6803 essentially as described by Cai & Wolk (1990). Total RNA was extracted according to Aiba *et al.* (1981), with slight modifications. For Northern blotting, 10 μg total RNA was denatured with glyoxal/DMSO, loaded on 0·8 % (w/v) agarose gels, and run in 10 mM sodium phosphate, pH 7 (Sambrook *et al.*, 1989). Transfer to nylon membranes, hybridization, washing and stripping were performed according to the membrane manufacturer's instructions (Millipore). Hybridization with the constitutively expressed *rnpB* gene (Vioque, 1992) was performed to estimate the loading of RNA samples. DNA probes were labelled by random priming using the Ready-To-GO kit (Amersham), using [α-^{32}P]dCTP. Blots were imaged using a Molecular Dynamics phosphorimager. DNA probes were as follows: probe 1, 80 bp *Hpa*I–*Spe*I (*cpcA–cpcC2* intergenic region) plus 350 bp *Nhe*I–*Hpa*I (*cpcC1–cpcD* intergenic region plus 200 bp of *cpcD*); probe 2, 600 bp from *Nae*I–*Hpa*I (*cpcC1* to *cpcD*); probe K, 1·2 kb *Pst*I fragment from pUC4K; probe *rnpB*, 500 bp *Hin*dIII–*Eco*RI fragment containing *rnpB* from *Synechocystis* sp. strain PCC6803 (Vioque, 1992).

Construction of plasmids and mutants. Primers pcf and pcr (5′-GTAGGCTGTGGTTCCCTAG-3′ and 5′-CACACTCTCAACGGTTCCG-3′, respectively) were used for the PCR amplification of the *cpc* operon from *Synechocystis* sp. strain PCC6803 total DNA. Primers were designed according to the Cyanobase complete genome sequence. The 4·2 kb PCR product was digested with *Mfe*I and *Pst*I and cloned into *Eco*RI- and *Pst*I-digested pUC9. The resulting plasmid, denoted pCPC, carried a 4 kb fragment which contained the five genes of the *cpc* operon plus 480 bp upstream of the *cpcB* start codon and 260 bp downstream from the stop codon of the last gene, *cpcD*. Part or all of *cpcC2* and *cpcC1* were substituted by the Km cassette in the inactivation plasmids pC30K+, pC30K−, pC33K+ and pCBK+ (Table 1). Mutants 30f, 30r, 33 and CB were created by transformation of the wild-type *Synechocystis* sp. strain PCC6803 with each of these plasmids, respectively, followed by screening for Km^r colonies. pΔC2*Ω* was constructed starting from an *Eag*I-digested pCPC plasmid treated for 20 min with BAL31 nuclease, then *Spe*I digested, treated with the Klenow DNA polymerase and ligated; the resulting plasmid contained a 685 bp deletion in *cpcC2*. Subsequently, the Ω cartridge was inserted in the *Bse*RI site. Transforming CB and the wild-type with plasmid pΔC2*Ω* and screening for (Km^s, Sp^r) colonies resulted in Δ30D3 and D3, respectively. Transformation of the wild-type with pUCDΩ and screening for Sp^r colonies gave the D4 mutant. For trans-complementations of *cpcC1* and *cpcD*, pS1C1 and pS1D were respectively used to transform the appropriate strains (Table 1). pS1D was constructed to express *cpcD* from the *psbAII* promoter by inserting into pPSBA2 a 380 bp *Nde*I–*Bam*HI fragment containing *cpcD* plus a *Bam*HI fragment carrying the *aphI* gene (Fig. 1). The *cpcD*-containing fragment was amplified using the following oligonucleotides: fd (5′-GAATTCCATATGTTAGGTCAATCTTC-3′) and rd (5′-GGGATCCTGACTCGATGGCTATTC-3′). *Nde*I and *Bam*HI sites are underlined. pS1C1 was constructed to express *cpcC1* from the *psbAII* promoter by inserting a 3·3 kb *Hin*cII fragment from 30 bp upstream of the *cpcC1* start codon (containing the ribosome-binding site) to 35 bp downstream of the *cpcC1* stop codon plus the Ω cassette in pPSBA2 (Fig. 1).

Plasmids constructed by the cloning of PCR products were sequenced to verify the fidelity of the PCR amplification. Complete segregation of the mutant alleles was confirmed by PCR, and in some cases the PCR products were subjected to restriction analysis to ascertain the identity of the amplified fragments (data not shown).

PBS isolation and analysis. Cells were broken by vortexing with glass beads. Phycobilisomes were prepared from *Synechocystis* sp. strain PCC6803 as described by Ajlani *et al.* (1995) and Glazer (1988). Absorption spectra were recorded on a Varian Cary-5E double-beam spectrophotometer, with a data interval of 0·5 nm. Proteins were analysed by SDS-PAGE on a 10–20 % linear polyacrylamide gradient in a modified Tris/glycine buffer (Fling & Gregerson, 1986) or 4–12 % Bis-Tris gels (NuPAGE, Novex) in MES-SDS buffer. OD at 620 nm was used to ensure approximately equal loading of different PBS samples; about 0·3 OD_{620} ml (100 μl from a sample at $OD_{620}=3$) was loaded per well. PBS-containing samples were concentrated by precipitation with 10 % (w/v) trichloroacetic acid prior to loading. Proteins were visualized using Coomassie Brilliant Blue stain.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Properties	Source or reference
Strains		
WT	Wild-type strain of *Synechocystis* sp. strain PCC6803	Pasteur Culture Collection
30f	Δ*cpcC2*::*aphI* – deletion of 68 % of *cpcC2* (codon 21–208), *aphI* insertion (Km^r)	This work
30r	Δ*cpcC2*::*aphI* – as above but *aphI* in the opposite orientation (Km^r)	This work
Δ30D3	Δ*cpcC2*, *cpcD*$^-$::Ω – deletion of 83 % of *cpcC2* (codon 2–230) and termination of the *cpc* operon transcription before *cpcD* (Sp^r, Sm^r)	This work
D3	*cpcD*$^-$::Ω – Ω insertion between *cpcC1* and *cpcD*, transcription termination before *cpcD* (Sp^r, Sm^r)	This work
33	Δ*cpcC1*::*aphI* – deletion of 50 % of *cpcC1* (codon 77–220), *aphI* insertion (Km^r)	This work
CB	Δ*cpcC2C1*::*aphI* – total deletion of *cpcC2* and *cpcC1* (codon 2 of *cpcC2* to the last codon of *cpcC1*), *aphI* insertion (Km^r)	This work
33c	33 transcomplemented with *cpcC1* in the *psbAII* locus (Km^r, Sp^r, Sm^r)	This work
CBc	CB transcomplemented with *cpcC1* in the *psbAII* locus (Km^r, Sp^r, Sm^r)	This work
D4	*cpcD*$^-$::Ω – Ω insertion in *Hin*cII of *cpcD*, transcription termination within *cpcD* (Sp^r, Sm^r)	This work
WD	WT plus *cpcD* in the *psbAII* locus (Km^r)	This work
D3D	D3 plus *cpcD* in the *psbAII* locus (Km^r, Sp^r, Sm^r)	This work
D4D	D4 plus *cpcD* in the *psbAII* locus (Km^r, Sp^r, Sm^r)	This work
Plasmids		
pCPC	4 kb *Mfe*I–*Pst*I genomic fragment carrying the *cpc* operon (Ap^r)	This work
pC30K+	Derivative of pCPC with Km cassette inserted between *Xho*I–*Eag*I (Km^r, Ap^r)	This work
pC30K−	As above with *aphI* gene in an opposite orientation (Km^r, Ap^r)	This work
pΔC2Ω	2·7 kb *Age*I–*Pst*I fragment carrying part of the operon minus 685 nt deletion from *cpcC2*. Ω was inserted 35 bp downstream of *cpcC1* (Ap^r, Sm^r/Sp^r)	This work
pC33K+	Derivative of pCPC with Km cartridge inserted between *Msc*I–*Nae*I (Km^r, Ap^r)	This work
pCBK+	Derivative of pCPC with Km cartridge inserted between *Spe*I–*Nhe*I (Km^r, Ap^r)	This work
pUCDΩ	*Nhe*I–*Pst*I fragment carrying *cpcD* with Ω inserted at *Hin*cII site (Ap^r)	This work
pPSBA2	500 bp upstream and 500 bp downstream of the *psbAII* ORF separated by a multiple cloning site cloned in pSL1180 (Ap^r)	Lagarde *et al.* (2000)
pS1D	*cpcD* and the Km cartridge inserted in the multiple cloning site of pPSBA2 (Ap^r, Km^r)	This work
pS1C1	*cpcC1* and the Ω cartridge inserted in the multiple cloning site of pPSBA2 (Ap^r, Sm^r/Sp^r)	This work
pUC4K	Origin of the Km^r cassette containing the *aphI* gene (Amp^r, Km^r)	Oka *et al.* (1981)
pDW9	Origin of the Ω fragment containing the *aadA* gene plus strong translation-transcription terminators on both sides (Amp^r, Sm^r/Sp^r)	Golden & Wiest (1988); Prentki & Krisch (1984)

RESULTS

Characterization of L_R^{30}-deficient strains

Three different strategies were used to inactivate *cpcC2*, encoding the L_R^{30} linker, in order to estimate any polar effect on flanking genes. The 30f and 30r strains carried a deletion in *cpcC2* that was replaced by opposite orientations of the Km cassette. The Δ30D3 strain contained a deletion in *cpcC2* plus the Ω cartridge inserted between *cpcC1* and *cpcD*. A control strain, designated D3, contained only the Ω insertion at the same location (Fig. 1 and Table 1).

The PBSs from each of the mutants were purified in order to determine their polypeptide composition. Sucrose gradients from all the PBS preparations contained a dark-blue band in the 1 M sucrose layer at positions near those expected for a complete PBS, indicating that PBSs were assembled. However, the assembled particles were at slightly higher positions on the sucrose gradient than those of the wild-type and the D3 strain, which indicated that the absence of L_R^{30} resulted in the assembly of a smaller PBS. The reduction of the PBS size is accompanied by the appearance of a free PC band at the top of the gradient tubes in the 0·25 M sucrose layer, indicating that a certain amount of synthesized PC is not incorporated into rods. Less free PC was found in 30r than in 30f and Δ30D3. Fluorescence emission upon excitation with biliprotein-sensitizing light showed high emission from PC in *cpcC2*-deficient cells compared to the wild-type and D3, confirming

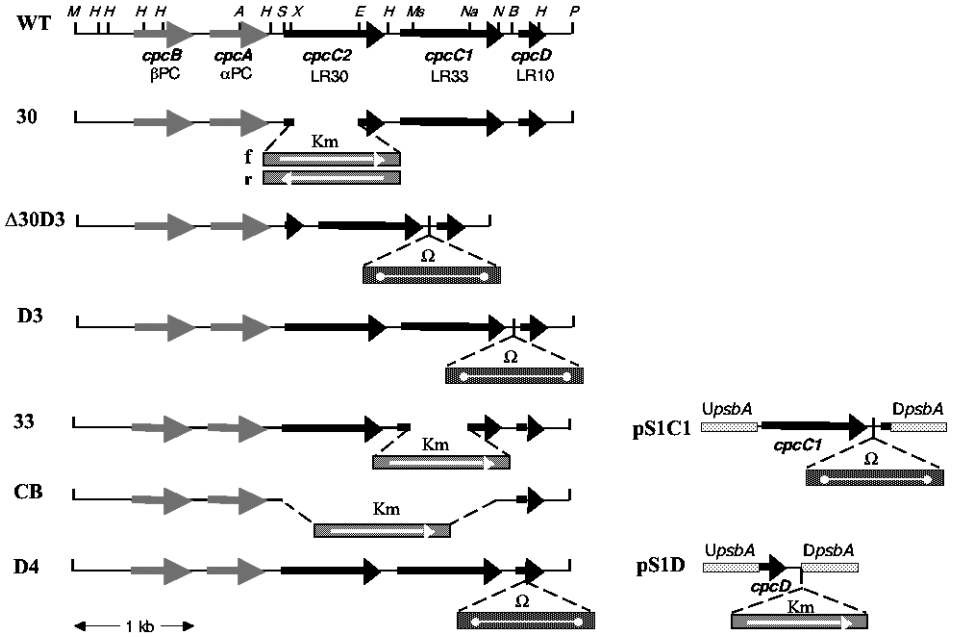

Fig. 1. Restriction and genetic maps of the *cpc* operon in *Synechocystis* sp. strain PCC6803 strains. WT, wild-type *cpc* operon; 30, 560 bp *Xho*I–*Eae*I fragment from *cpcC2* replaced by the Km cassette (f and r indicate orientations of the *aphI* gene transcription in the respective strains); Δ30D3, 685 bp deleted from *cpcC2* plus Ω cassette inserted in the *Bse*RI site; D3, Ω cassette inserted in the *Bse*RI site; 33, 425 bp *Msc*I–*Nae*I fragment from *cpcC1* replaced by the Km cassette; CB, 1·7 kb *Spe*I–*Nhe*I fragment containing both *cpcC2* and *cpcC1* replaced by the Km cassette; D4, Ω cassette inserted in the *Hin*cII site of *cpcD*; pS1C1, transcriptional fusion of *cpcC1* to the *psbAII* promoter; pS1D, translational fusion of the *cpcD* ORF to the *psbAII* initiation codon. U*psbA*, upstream *psbAII*; D*psbA*, downstream *psbAII*. Phycocyanin subunits encoding genes are represented as grey arrows, rod-linker encoding genes as black arrows, Ω and the Km cassettes as white lines in a dark rectangle; an arrowhead indicates the direction of gene transcription. Abbreviations: *A*, *Age*I; *B*, *Bse*RI; *E*, *Eae*I; *H*, *Hin*cII; *M*, *Mfe*I; *Ms*, *Msc*I; *Na*, *Nae*I; *N*, *Nhe*I; *P*, *Pst*I; *S*, *Spe*I; *X*, *Xho*I.

the presence of unincorporated PC in these strains (data not shown).

The polypeptide composition of purified PBSs from the mutants was analysed by Coomassie-stained SDS-PAGE. In addition to the phycobiliproteins (PC and AP) in the 20 kDa range, wild-type PBSs contain seven polypeptides (Fig. 2a, lane WT). Four of these polypeptides, not encoded by the *cpc* operon, were invariably present in all strains: L_C, core linker; L_{RC}, rod-core linker; FNR, Ferredoxin $NADP^+$ Reductase and L_{CM}, core-membrane linker. As expected, the L_R^{30} polypeptide was absent from the three *cpcC2*-deleted strains, Δ30D3, 30f and 30r. At the same time, these strains exhibited decreasing amounts of the L_R^{33} polypeptide (traces, if any, in 30r) (Fig. 2a). The L_R^{10} polypeptide was detected only in the 30f PBS, albeit in lesser amount than in the wild-type PBS.

The purified PBSs from the mutants were further characterized by comparing their absorption spectra to that of the wild-type PBS. In wild-type PBS, PC ($\lambda_{max}=620$ nm) is the major phycobiliprotein (~75 %). PBSs from the *cpcC2*-deleted strains all exhibit lower amounts of incorporated PC. Consequently, the contribution of AP ($\lambda_{max}=650$ nm) to the PBS absorption spectrum becomes evident. PBSs from 30r had a spectrum corresponding to the lowest relative amount of PC and 30f had an intermediate amount, while Δ30D3 PBS contained the largest amount of PC (Fig. 2b). The increasing amounts of PC incorporated into the PBSs of the three allelic mutants, 30r, 30f and Δ30D3, were proportional to the amount of the L_R^{33} polypeptide detected in their PBSs (Fig. 2a). Since L_R^{33} is the product of *cpcC1* located downstream of the inactivated *cpcC2* gene, the different amounts of L_R^{33} might be due to different polar effects of the *cpcC2* mutations on *cpcC1* expression.

The steady-state level of *cpc* transcripts was monitored in the mutants by Northern blot. The wild-type operon specifies three differently sized mRNAs which start upstream of the translation initiation codon of *cpcB* and terminate at three different sites (Fig. 3a). The smaller and most abundant transcript (1·6 kb), containing *cpcB* and *cpcA*, does not seem to be affected in any of the mutants (Fig. 3b, panel 1). Two larger and less-abundant transcripts, which contain the linker-encoding genes *cpcC2*, *cpcC1* and *cpcD*, are detected at 3·4 and 3·8 kb in the wild-type (Fig. 3b, panels 1 and 2, lane WT). The size and relative abundance of these transcripts are changed in the mutants. In the 30r and 30f

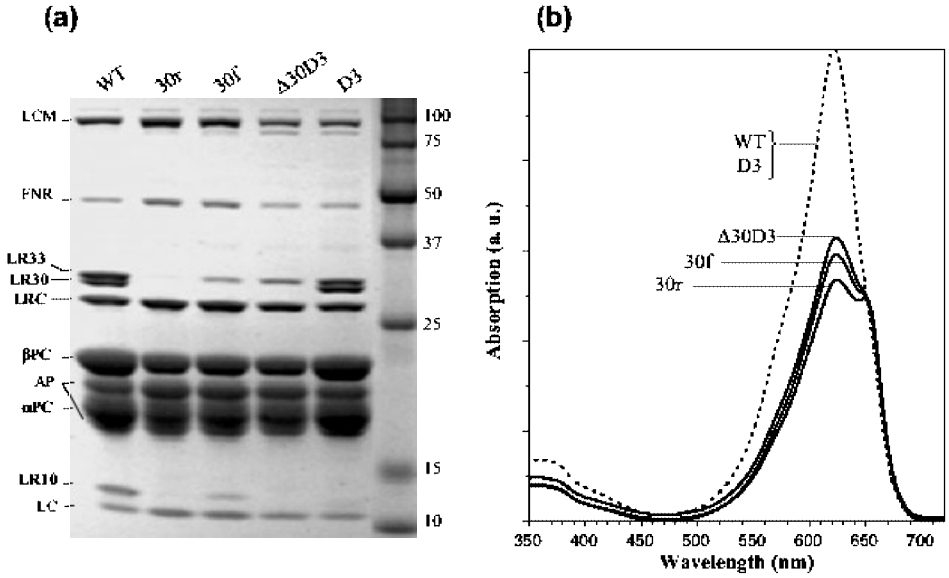

Fig. 2. (a) Polypeptide composition of purified PBS from wild-type, WT, and from rod-linker-deficient strains 30r, 30f, Δ30D3 and D3 analysed by SDS-PAGE (10–20 % linear acrylamide gradient in Tris/HCl). The identities of polypeptides are labelled on the left. Masses of the molecular marker are indicated in kDa on the right. (b) Absorption spectra of purified PBSs from wild-type and rod-linker-deficient strains. Wild-type and D3 PBSs have similar absorption spectra (λ_{max}, 620 nm), the lower amounts of PC incorporated in the Δ30D3, 30f and 30r PBSs revealed the contribution of AP (λ_{max}, 650 nm) to their absorption spectra. The decreasing height of the 620 nm peaks reflects the decreasing amount of PC in the PBS. The spectra were normalized to the 655 nm absorption.

strains, they would be about 0·6 kb larger due to the deletion in *cpcC2* and the Km cassette insertion; their sizes should be 4·0 and 4·4 kb, respectively. They were undetectable in the 30r strain and present in a drastically reduced amount in strain 30f (Fig. 3b, panel 2). In D3, the 3·8 kb transcript is absent due to the strong transcription terminators present in Ω, while the 3·4 kb transcript seems less abundant than in the wild-type. This might be due to a stem–loop disturbed by the Ω insertion (see Discussion). In Δ30D3, a shorter form of this transcript (2·7 kb due to the deletion in *cpcC2*) is detected and seems fairly abundant. The blot was hybridized with a Km probe and overexposed to detect mRNAs containing the Km cassette (Fig. 3b, panel K). In 30f, two major transcripts are detected at 4·0 and 2·9 kb, while in strain 30r, the 4·0 kb one was barely detectable and the 2·0 kb transcript that also hybridized to probe 1 was obvious. Larger bands (around 2·5 kb) could be the products of abortive transcription and degradation of the full operon transcript. The 4·0 kb transcripts correspond to the transcription of the whole operon through the Km cassette. Their low amounts demonstrate that the insertion of the Km cassette destabilizes those transcripts. The 2·9 and 2·0 kb transcripts, in 30f and 30r, respectively, are the products of readthrough transcription from the *aphI* promoter. The 2·9 kb transcript detected in 30f terminates at the end of the *cpc* operon: it should then contribute to the translation of *cpcC1* and *cpcD*. The size of the 2 kb transcript in 30r implies that it terminates within *cpcB*. Part of this transcript is an antisense RNA to the 1·6 kb mRNA containing *cpcB* and *cpcA*.

Characterization of the L_R^{33}-deficient strain

cpcC1, encoding the L_R^{33} linker, was inactivated by replacing an internal fragment with the Km cassette; the resulting mutant was denoted 33. A strain in which both *cpcC2* and *cpcC1* genes were totally deleted and replaced by the Km cassette was also constructed and denoted CB (Fig. 1 and Table 1).

Sucrose gradients of PBS preparations from 33 and CB contained a dark-blue band at a position significantly higher on the gradient than those of the wild-type PBS, indicating that assembled PBSs were much smaller in size. Again, free PC bands at the top of the gradients indicate the presence of unincorporated PC in the cells. The SDS-PAGE of purified PBSs showed no trace of either L_R^{33} or L_R^{30} in CB. More surprisingly, both linkers were also absent in strain 33. The relative amounts of L_R^{10} were also significantly reduced in both mutants (Fig. 4a, lanes 33 and CB). PBSs from 33 and CB have identical absorption spectra, similar to the spectrum of 30r (Fig. 4b). The absence of L_R^{30} in the PBS from strain 33 suggests that L_R^{30} was not incorporated into the PBS in the absence of L_R^{33}. In order to corroborate this hypothesis and to exclude any polar effect of the *cpcC1* mutation, both CB and 33 were complemented with a transcriptional fusion in which *cpcC1* was

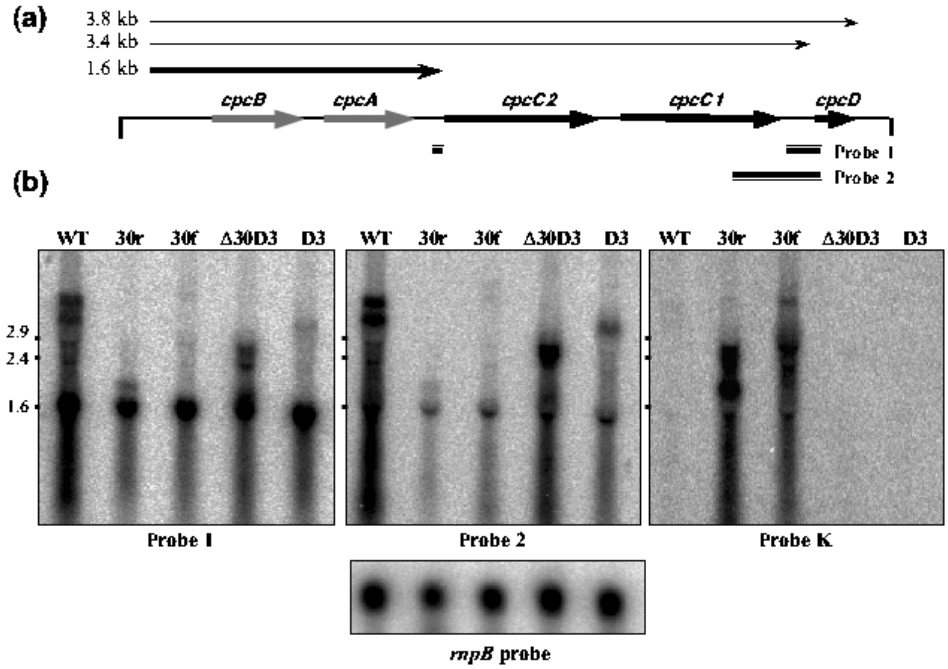

Fig. 3. (a) The three mRNAs produced from the *cpc* operon in *Synechocystis* sp. strain PCC6803. Positions of two of the DNA probes used in panel (b) are shown below a map of the operon. (b) Northern-hybridization analysis of the *cpc* operon in the wild-type and four rod-linker-deficient strains. DNA probes were chosen to be general for all the *cpc* transcripts (probe 1), specific for the large *cpc* transcripts (probe 2), and specific for the Km cassette containing transcripts (probe K); the *rnpB* gene was used to estimate the loading of the samples. Approximately 10 μg total RNA was loaded per lane (4 mm well width). The same blot was used for all four hybridizations. Positions of the rRNAs are indicated on the left of each blot. Note that signal clouds are detected at the positions of the rRNA where degradation products of larger RNA molecules are trapped.

placed under the control of the strong *psbAII* promoter in the *psbAII* locus (Fig. 1, pS1C1). The resulting strains were denoted CBc and 33c, respectively. This insertion is neutral to *Synechocystis* sp. strain PCC6803, since it has been shown that *psbAIII* can support photoautotrophic growth in the absence of *psbAII* (Mohamed & Jansson, 1989). The SDS-PAGE showed that CBc PBSs recovered L_R^{33}, demonstrating that the ectopic *cpcC1* gene was expressed and capable of complementing the deleted gene. PBSs from 33c recovered both $L_R^{33}$and L_R^{30}, confirming that the absence of both linkers in 33 was due only to the deletion of *cpcC1* (Fig. 4a). Absorption spectra showed increased amounts of PC in CBc and 33c PBSs compared to their parent mutants CB and 33, respectively. The extent of this increase was more spectacular in 33c, since it recovered two rod linkers and their associated PC (Fig. 4b). These results prove that *cpcC2* was functional in strain 33; the absence of its product, L_R^{30}, in the PBS was due to an epistatic effect of *cpcC1* on *cpcC2*. It is noteworthy that the relative amounts of L_R^{10} also increased in the PBSs of 33c compared to CB, CBc and 33 (Fig. 4a).

Characterization of L_R^{10}-deficient and trans-complemented strains

The D3 strain (described in the L_R^{30} section) was indistinguishable from the wild-type with respect to growth rate and fluorescence emission characteristics. The polypeptide profile of D3 PBS confirmed the absence of the L_R^{10} linker and revealed a small decrease in the L_R^{33} to L_R^{30} ratio (Fig. 2a). Close examination of the D3 PBS absorption spectrum revealed a higher contribution from AP (shoulder at 650 nm), which means that the D3 PBS contained less PC (Fig. 5b). In this mutant, the Ω cassette was inserted upstream of *cpcD*, destroying a stem–loop structure that might stabilize the 3·4 kb mRNA where *cpcC1* is at the 3′ end. In order to verify this possibility, a mutant was created by inserting the Ω cassette in the *cpcD* ORF. PBS from this mutant, denoted D4, lacked L_R^{10} but contained a normal L_R^{33} to L_R^{30} ratio, and the absorption spectra were identical to that of the wild-type (Fig. 5a). Therefore, the decrease in L_R^{33} and the modified absorption spectra observed in D3 were due to the absence of the stem–loop in the 3·4 kb mRNA (see Discussion). Similar results were obtained in *Synechococcus* sp. strain PCC7002 mutants in which the stem–loop between *cpcC* and *cpcD* was deleted (de Lorimier *et al.*, 1990a).

Since the loss of L_R^{10} produced no detectable phenotype, we attempted the overexpression of *cpcD* to shed light on its function. A fragment starting at the *cpcD* initiation codon and ending downstream of its transcription terminator was used to produce a construct placing *cpcD* under the

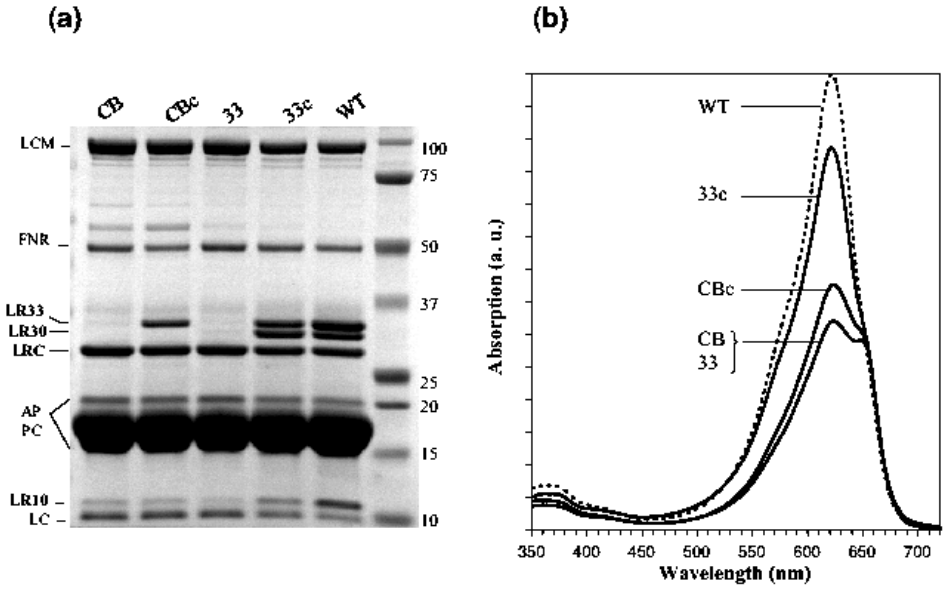

Fig. 4. (a) Polypeptide composition of purified PBS from wild-type, WT, rod-linker-deficient and complemented strains CB, CBc, 33, 33c analysed by SDS-PAGE (NuPage 4–12 % acrylamide gradient in Bis-Tris/HCl). The identities of polypeptides are labelled on the left. Masses of the molecular marker are indicated in kDa on the right. (b) Absorption spectra of purified PBSs from wild-type, rod-linker-deficient and complemented strains; the absorption spectra of CB and 33 contained the lowest amounts of PC (λ_{max}, 620 nm). The increasing height of the 620 nm peaks reflects the increasing amount of PC in the PBSs of the complemented strains CBc and 33c. The spectra were normalized to the 655 nm absorption.

control of the *psbAII* transcriptional and translational signals in plasmid pS1D (Fig. 1). Transformation of the wild-type with this construct resulted in strain WD. This strain produced a high level of a 0·4 kb mRNA containing *cpcD*, indicating that the ectopic *cpcD* was transcribed at the level expected from the *psbAII* promoter (Northern

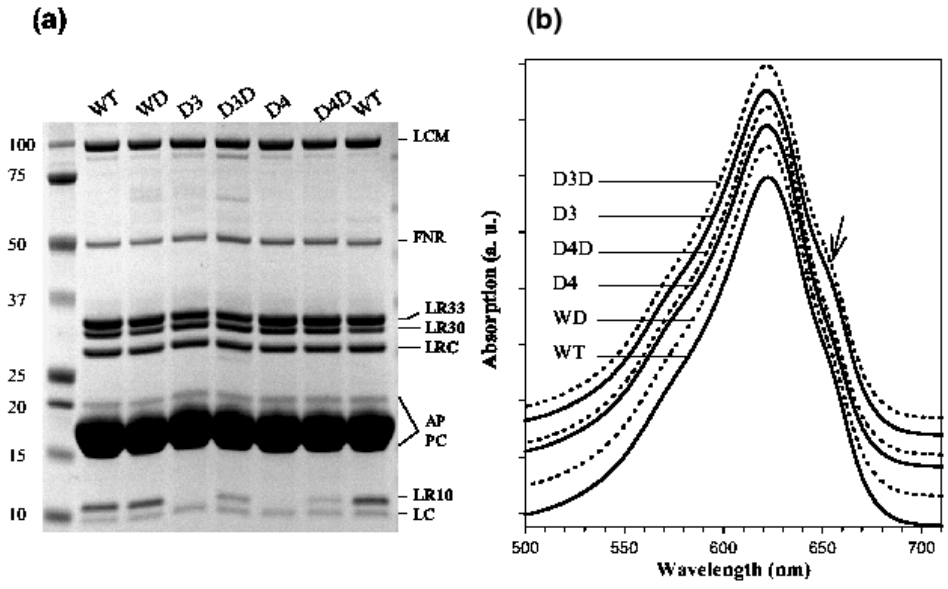

Fig. 5. (a) Polypeptide composition of purified PBSs from *cpcD* mutant strains analysed by SDS-PAGE (NuPage 4–12 % acrylamide gradient in Bis-Tris/HCl). The identities of polypeptides are labelled on the right. Masses of the molecular marker are indicated in kDa on the left. (b) Absorption spectra of purified PBSs. The contribution of AP (arrow at 650 nm) was more obvious in the D3 and D3D spectra. The spectra were shifted for better viewing.

blot not shown). PBSs from WD were indistinguishable from those of the wild-type regarding both their polypeptide composition and their absorption spectra (Fig. 5a, b). In order to estimate the production of L_R^{10} polypeptide from the ectopic *cpcD* gene, the *cpcD* deficient strains D3 and D4 were transformed with pS1D, resulting in strains D3D and D4D, respectively. PBSs from both strains recovered L_R^{10}, but in much lower amounts than the wild-type (Fig. 5a). This result indicates that although the ectopic gene overproduced an mRNA containing *cpcD*, the L_R^{10} polypeptide was not proportionally translated or was degraded before its incorporation into the PBS. We believe that when L_R^{10} is produced *in trans*, proteolysis prevents its association to the PBS: it is known that linker polypeptides are highly sensitive to proteolysis when they are not associated to phycobiliproteins. Therefore, overexpression of a linker polypeptide should be achieved by placing the ectopic gene as close as possible to the *cpc* operon, as was carried out in *Synechococcus* sp. strain PCC7942 (Bhalerao & Gustafsson, 1994), although these authors did not check the ability of their ectopic *cpcD* gene to complement a *cpcD*-deficient strain.

DISCUSSION

In this study, we have constructed and characterized ten mutants with different modifications in three PBS rod-linker genes, located in the *cpc* operon of *Synechocystis* sp. strain PCC6803. This operon contains five genes and produces three transcripts from one transcription initiation site. The site was mapped 251 bp upstream from the translation start for *cpcB* in *Synechocystis* sp. strain PCC6714 (Imashimizu *et al.*, 2003), a strain that is closely related to *Synechocystis* sp. strain PCC6803. Sequence analysis identified a stable stem–loop structure downstream from *cpcA* which might act as a transcriptional terminator (not followed by a polyT stretch) or as a 3′ stabilizer to an abundant 1·6 kb transcript containing *cpcB* and *cpcA*. This structure was intact in all mutants, which explains the constant amount of this transcript in the different strains. Less-abundant 3·4 and 3·8 kb transcripts containing *cpcC2*, cpc*C1* and cpc*D*, in addition to *cpcB* and *cpcA*, appear to be generated by a readthrough of the above RNA structure and two rho-independent terminations downstream of *cpcC1* and *cpcD*, where significant stem–loops and polyT stretches are detected. The large transcript containing *cpcD* was absent in both D3 and Δ30D3 due to transcription termination at the Ω cassette edge. The fact that the 3·4 kb transcript was less abundant in D3 suggests that the Ω terminator does not provide a 3′ stabilizer effect. The reduced levels of the larger transcripts in strains 30f and 30r explain the decrease of L_R^{33} and L_R^{10} in their PBSs (Fig. 3b, panel 2). The detected readthrough transcripts from the Km cassette (*aphI* gene) in strain 30f (Fig. 3b, panel K) should contribute to the translation of genes downstream of the insertions *cpcC1* and *cpcD*. The lower amount of L_R^{33} and L_R^{10} in 30f PBS (Fig. 2a) suggests that the transcripts initiating from the *aphI* promoter are less abundant or are unstable. In the 30r strain, the Km cassette severely disturbed the full operon transcript (larger transcripts are barely detectable) and produced an antisense RNA to the 1·6 kb transcript, which explains the lower PC content in this strain. Note that this *aphI* transcript terminates in *cpcB* (where a significant stem–loop structure and a polyT stretch are detected), and not at the stem–loop structure downstream of *cpcA*. This observation suggests that the latter structure is an RNA-processing site, rather than a terminator. This possibility was evoked for the *cpc* operon of *Anabaena* PCC7120 (Belknap & Haselkorn, 1987).

The three allelic *cpcC2* mutants, 30r, 30f and Δ30D3, contained trace, reduced and almost-normal amounts of L_R^{33}, respectively (Fig. 2a). We showed that this was due to different expression levels of *cpcC1*. This gene undergoes a polar effect from the Km cassette inserted upstream in 30f and 30r and from the downstream Ω cassette insertion in Δ30D3. These polar effects resulted in a decreased amount of the *cpcC1* product, L_R^{33}; consequently the amount of PC incorporated in the PBS decreased. This result suggests that transcriptional regulation of the rod-linker genes could adjust the light-harvesting capacity of the PBS by modulating the amounts of PC incorporated into the rods. Decrease of the level of a larger transcript relative to a smaller one in response to high light levels has been shown to occur in the *cpc* operon of *Anabaena* PCC7120 (Belknap & Haselkorn, 1987). The relative level of the transcripts could be modulated through transcription termination at the stem–loop downstream of *cpcA* or by RNA processing at this site followed by degradation of the linker-encoding mRNA. In *Synechocystis* sp. strain PCC6803 and *Synechococcus* sp. strain PCC6301 PBSs, the L_R^{30} to L_R^{33} ratio was repeatedly <1, decreasing under high-light conditions (our unpublished observations; Lönneborg *et al.*, 1985). In view of the *cpcC2* and *cpcC1* co-transcription, one must imagine a form of regulation in which *cpcC2* is down-regulated first. Endoribonuclease processing downstream of *cpcA* followed by 5′→3′ progressing degradation (Rauhut & Klug, 1999) of the linker-encoding RNA could accomplish this, since *cpcC2* would be down-regulated first and *cpcC1* would be protected by the 3′ stem–loop structures. Further experiments are needed to test this hypothesis.

The CB mutant is similar to the An112 mutant of *Synechococcus* sp. strain PCC6301 (Yamanaka *et al.*, 1980) and the PR6009 mutant of *Synechococcus* sp. strain PCC7002 (de Lorimier *et al.*, 1990b). All these mutants show that L_{RC} is involved in association of the core-proximal PC hexamers, at the base of every rod, with the core. Two modes of assembly are then possible for the remaining PC hexamers: (i) L_R^{33} and L_R^{30} are interchangeable – either can be present in both the intermediary and distal PC hexamers; (ii) the intermediary PC hexamers contain only one linker type, with the other specifically present in the distal PC hexamers. Careful characterization of mutants deficient in each of these linkers should favour one of these modes. Such

mutants constructed in *Synechococcus* sp. strain PCC7942 suggested that L_R^{30} could replace L_R^{33} in its absence (Bhalerao *et al.*, 1991, 1993), favouring the first mode but in contradiction with the results presented here. Indeed, the first mode was excluded by two of our observations: (i) the absence of L_R^{33} prevented L_R^{30} attachment in the *cpcC1*-deficient strain 33, and (ii) transcomplementation of strain 33 with *cpcC1* restored attachment of both L_R^{33} and L_R^{30}. We therefore favour the second mode, with L_R^{33} attaching the intermediary PC hexamer and L_R^{30} attaching the distal one. This mode is also in agreement with results obtained upon nitrogen starvation, where sequential loss of PC and associated linkers occurs. L_R^{30} disappears first from the PBS of *Synechocystis* sp. strain PCC6803 and *Synechococcus* sp. strain PCC6301 during the initial steps of nitrogen starvation, suggesting that this linker is associated with the peripheral PC hexamer (Richaud *et al.*, 2001; Yamanaka & Glazer, 1980). In terms of genetics, *cpcC2* and *cpcC1* might have been created by gene duplication in an ancestral strain that contained only one *cpcC*, such as *Synechococcus* sp. strain PCC7002. The resulting genes then acquired different functions, and *cpcC1* became epistatic to *cpcC2*. The domain that determines the specificity of L_R^{30} and L_R^{33} may lie within the C-terminal third of their sequences, since it is less conserved than the remaining two-thirds (28 % and 55 % identity for each, respectively). Specific interactions between these rod linkers seem to exclude their random insertion in *Synechocystis* sp. strain PCC6803 but not in mutants of *Synechococcus* sp. strain PCC7942. Amino acid sequence comparison of L_R^{33} and L_R^{30} from *Synechocystis* sp. strain PCC6803 and *Synechococcus* sp. strain PCC7942 did not provide an explanation for the differing results obtained in the two strains.

L_R^{10} was present, albeit in smaller amounts, in PBSs lacking the core-distal (30f) or both the intermediary and the core-distal PC hexamers (33 and CB). Its reduced level was first attributed to the polar effect of the Km cassette on *cpcD* expression in these strains, but the increased amounts of L_R^{10} in the 33c PBS compared to CBc (Fig. 4a) suggests that, although the polar effect exists, L_R^{10} might have a higher affinity for a PC hexamer containing L_R^{30}. Indeed L_R^{10} was proposed to be associated with the distal end of the rods (de Lorimier *et al.*, 1990a). Its absence does not seem to affect PBS assembly or function in *Synechocystis* sp. strain PCC6803.

In cyanobacteria, the FNR contains an N-terminal domain that shares similarity to PBS linker polypeptides (Schluchter & Bryant, 1992). The highest similarity was found to the L_R^{10} linker (about 32 % identity and 75 % similarity over 80 amino acids), while lower similarities were found to the C-terminal domains of L_R^{33} and L_R^{30}, and even to the core-associated linker L_C. It has been proposed that the FNR shares the same binding site as L_R^{10} in the PBS rod of *Synechococcus* sp. strain PCC7002 (Gomez-Lojero *et al.*, 2003). If they shared the same binding site, one would expect the relative amounts of FNR to increase in the absence of L_R^{10}, which was not observed in any of the mutants constructed here. The binding site of the FNR has also been suggested to be the core-proximal PC hexamer in *Synechocystis* sp. strain PCC6803 (van Thor *et al.*, 1999), but more direct evidence is needed in order to localize the linkers and the FNR in the PBS.

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